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Published in:
Autophagy

[Autophagy 3:2, 96-105; March/April 2007]; ©2007 Landes Bioscience

Research Paper

A Peroxisomal Lon Protease and Peroxisome Degradation by Autophagy Play Key Roles in Vitality of *Hansenula polymorpha* Cells

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Original manuscript submitted: 10/10/06
Manuscript accepted: 10/23/06

Previously published online as an *Autophagy* E-Publication:
http://www.landesbioscience.com/journals/autophagy/abstract.php?id=3534

KEY WORDS

peroxisome, autophagy, Lon protease, yeast, housekeeping

ABBREVIATIONS

ROS	reactive oxygen species
ERAD	endoplasmic reticulum associated degradation
ER	endoplasmic reticulum
PMSF	phenylmethylsulfonylfluoride
H2DCFDA	dichlorodihydrofluorescein diacetate
DHFR	dihydrofolate reductase

ACKNOWLEDGEMENTS

We gratefully acknowledge the assistance of Anjuman Begum, Nancy Halsema, Arjen Krikken, Klaas Sjollema, Willem van der Poll and Patricia Stevens in different parts of the work. We thank Prof. Dr. Thomas Langer (Köln, Germany) for providing the original DHFR plasmids. E.Bener Aksam is supported by NWO/ALW. I.J. van der Klei holds a PIONIER fellowship (NWO), which also supports A. Koek.

ABSTRACT

In eukaryote cells various mechanisms exist that are responsible for the removal of non-functional proteins. Here we show that in the yeast *Hansenula polymorpha* (*H. polymorpha*) a peroxisomal Lon protease, Pln, plays a role in degradation of unfolded and non-assembled peroxisomal matrix proteins. In addition, we demonstrate that whole peroxisomes are constitutively degraded by autophagy during normal vegetative growth of WT cells.

Deletion of both *H. polymorpha PLN* and *ATG1*, required for autophagy, resulted in a significant increase in peroxisome numbers, paralleled by a decrease in cell viability relative to WT cells. Also, in these cells and in cells of *PLN* and *ATG1* single deletion strains, the intracellular levels of reactive oxygen species had increased relative to WT controls. The enhanced generation of reactive oxygen species may be related to an uneven distribution of peroxisomal catalase activities in the mutant cells, as demonstrated by cytochemistry.

We speculate that in the absence of HpPln or autophagy unfolded and non-assembled peroxisomal matrix proteins accumulate, which can form aggregates and lead to an imbalance in hydrogen peroxide production and degradation in some of the organelles.

INTRODUCTION

Cellular housekeeping is important for optimal performance of cells/organisms and prevents the accumulation of abnormal, unwanted proteins by a variety of mechanisms. Non-functional proteins include newly synthesized misfolded or non-assembled proteins, but also proteins that are damaged, for instance due to oxidation by reactive oxygen species (ROS).[1] Examples of housekeeping processes are the protein folding functions of molecular chaperones and protein degradation processes. Housekeeping mechanisms have been established in most cellular compartments in eukaryote cells. For instance, in mitochondria chaperones of the hsp60 and hsp70 protein families are involved in folding and assembly of newly synthesized proteins and mitochondrial proteases degrade non-functional proteins.[2,3] In the cytosol the ubiquitin proteasome system selectively degrades redundant and malfunctional proteins, whereas ERAD is responsible for the elimination of such proteins from the lumen of the endoplasmic reticulum (ER).[4,5] Lysosomes/vacuoles contribute to cellular housekeeping by degrading cytoplasmic components, a process termed autophagy.[6]

So far, little is known of housekeeping processes related to peroxisomes. Chaperones of the hsp60 or hsp70 protein families are not generally encountered in these organelles. Because most peroxisomal proteins are folded and assembled prior to import, chaperones involved in these processes are most likely mainly cytosolic. Also, reports on peroxisomal proteases are rare and their functions still speculative.[7-10] The fate of non-functional peroxisomal proteins that have entered the organelle or that have become damaged in the organelle matrix is largely unknown. A process that has been studied in more detail is the degradation of whole peroxisomes by autophagy, which is for instance induced when cells experience nitrogen depletion. At these conditions portions of the cytoplasm (including cell organelles) are recycled in the vacuole/lysosome.

Peroxisomes can also be selectively degraded by autophagy (a process also designated pexophagy). Selective peroxisome degradation has been extensively studied in yeast and is induced when peroxisomes become redundant for growth (e.g., upon a shift from peroxisome inducing to peroxisome repressing growth conditions).[11]

Here we report on the identification of a peroxisomal Lon protease in the yeast *Hansenula polymorpha* (*H. polymorpha*). Lon proteases belong to the family of AAA-ATPases.[3] Members of this protein family are commonly present in bacteria and

mitochondria.[3] Recently, a Lon protease was identified in rat peroxisomes, but the function of this protease has not been analysed yet.[7]

We also show that in the yeast *H. polymorpha* the peroxisomal Lon protease and degradation of peroxisomes by autophagy are important for cell vitality. The results of these studies are presented in this paper.

MATERIALS AND METHODS

Organisms and growth. The *H. polymorpha* strains and plasmids used in this study are listed in Tables 1 and 2. Cells were grown at 37°C or 45°C in batch or continuous cultures using mineral media as described previously.[12] These media contained 0.5% carbon source and 0.25% nitrogen source. Carbon-limited continuous cultures were grown at dilution rates of D = 0.06 h^{-1} or D = 0.1 h^{-1}. Leucine was added to the media at a final concentration of 30 $\mu g.ml^{-1}$. Heat shock treatment was performed essentially as described before.[13]

For the analysis of peroxisome turn-over in continuous cultures, cells containing the gene encoding GFP-SKL under control of the amine oxidase promoter (P_{AMO}) were used. Synthesis of the peroxisomal marker GFP-SKL was induced and subsequently fully repressed. In detail, cells were grown at a dilution rate of D = 0.06 h^{-1} until a steady state was reached (obtained after at least five volume changes). Then the feed of the culture was changed from medium containing methylamine as the sole nitrogen source (induction of P_{AMO}) to medium containing ammonium sulphate (repression of P_{AMO}). At the same time ammonium sulphate (final concentration 0.25%) was added to the culture to obtain immediate repression of P_{AMO}.

Yeast transformants were selected on YND plates containing 0.67% Yeast Nitrogen Base without amino acids (Difco), 1% glucose and 2% agar and appropriate amino acids.

Molecular techniques. Standard recombinant DNA techniques were carried out essentially as described by Sambrook.[14] Transformation of *H. polymorpha* was performed by electroporation as detailed previously.[15] Restriction enzymes and biochemicals were obtained from Roche Applied Sciences (Almere, The Netherlands) and used as detailed by the manufacturer. Southern blot analysis was performed using the ECL direct nucleic acid labeling and detection system (Amersham Corp., Arlington Heights, IL). Primers used for polymerase chain reactions are listed in Table 3.

Table 1 ***H. polymorpha* strains used in this study**

Strain	Relevant Properties	Reference
WT, *leu 1.1 ura3*	Wild type NCYC495, *leu 1.1 ura 3*	45
WT, *leu 1.1*	Wild type NCYC495, *leu 1.1* URA3	45
WT::P_{AMO} *GFP-SKL*	Wild type containing a single copy intergration of the *GFP-SKL* gene, behind the P_{AMO} promoter	19
atg1	*Atg1* disruption strain, *leu1.1*	18
atg1::P_{AMO} *GFP-SKL*	atg1 containing a single copy intergration of the *GFP-SKL* gene under control of the P_{AMO} promoter	This study
WT::P_{PLN}*GFP-PLN*	WT containing the replacement of *PLN* by *GFP.PLN*, under control of the *PLN* promoter.	This study
WT::P_{PLN}*GFP-PLN*:: P_{AOX}*DsRED-SKL*	WT::P_{PLN}*GFP-PLN* containing one copy integration of plasmid pHIPZ4-DsRED-SKL	This study
Pln	*PLN* disruption strain, *leu1.1*	This study
pln:: P_{AMO}*GFP-SKL*	*pln* containing a single copy intergration of the *GFP-SKL* gene, behind the P_{AMO} promoter	This study
WT::P_{AOX}DHFR-SKL	Wild type containing a single copy intergration of the *DHFR-SKL* gene, behind the P_{AOX} promoter	This study
WT::P_{AOX}*DHFR^{MUT}-SKL*	Wild type containing a single copy intergration of the *DHFR^{MUT}-SKL* gene behind the P_{AOX} promoter	This study
pln::P_{AOX}*DHFRMUT-SKL*	*pln* containing a single copy intergration of *DHFR^{MUT}-SKL* behind the P_{AOX} promoter	This study
pln.atg1	Double disruption strain of *pln* and *atg1*	This study

Table 2 ***H. polymorpha* plasmids used in this study**

Plasmid	Relevant Properties	Reference
pBSK$^+$	pBluescript II SK$^+$	Stratagene Inc., San Diego, CA
pHI1	*E. coli* vector with *H. polymorpha URA3* gene; amp^R	21
pHIPX4	Shuttle vector containing the P_{AOX} promoter	25
pHIPX5-GFP-SKL	pHIPX5 containing the *GFP* gene fused to the PTS1 signal -SKL	19
pHIPZ4-DSRed-SKL	pHIPZ4 containing a gene encoding DsRed fused to the tripeptide -SKL under control of the P_{AOX} promoter	22
SSJ006	pHI1-*GFP-PLN*	This study
SSJ000	pHI1 containing P_{PLN}*GFP-PLN*	This study
SSJ007	pBSK$^+$-*DHFR^{mut}-SKL*	This study
PJK001	pBSK$^+$-*DHFR-SKL*	This study
SSJ001	PHIPX4 containing *DHFR^{MUT}-SKL* behind the P_{AOX} promoter	This study
PJK002	PHIPX4 containing *DHFR-SKL* behind the P_{AOX} promoter	This study
pBS-PDD7-URA	pBluescript in which the *PDD7* gene was replaced by the *URA3* gene	18
pKVK34	pPBS-PDD7-1 without the *Sal*I fragment, amp^R	This study
pKVK35	plasmid containing the *ATG1* deletion cassette; amp^R.*Ca-LEU2*	This study
pBSK-URA3	pBluescript containing the *H. polymorpha URA3* gene	17

Construction of the various *H. polymorpha* strains. For disruption of the *H. polymorpha PLN* gene a disruption cassette was constructed by a PCR based strategy using the *H. polymorpha URA3* gene as selection maker.[16] To this purpose the 5' and 3' flanking regions of genomic *PLN* were amplified using the primers plnfw1/plnrev1 and plnfw2/plnrev2, respectively. The resulting products, plnfragment1 containing a 3' overhang of the *URA3* gene and plnfragment2 containing a 5' overhang of the *URA3* gene, were used as primers in a PCR reaction using plasmid pBSK-URA3 as a

Table 3 **Oligonucleotides used in this study**

Primer	Sequence
GFPplnfwA	5′-CTCGGCATGGACGAGCTGTACAAG ATGTCTTCCAAGTCTTCAG CGTG-3′
GFPplnfwB	5′-CGCGGATCCATGGTGAGCAAGGGCGAG3′
GFPplnrevA	5′-GCCGGTACCCAGTGCCGTATACAAATTCCG-3′
GFPplnfwC	5′-GTTCTTGCTGCCGTCGCATGCATCCGC-3′
GFPplnrevC	5′-CGCGGATCCGCTAGTGGAATATATCTGAC3′
plnfw1	5′-CTCGCCAAAAGTAGCCTTCAG-3′
plnrev1	5′-CATAATTGCGTTGCTGAACATCAGTTGAAGCTCCGGTCGTCTC GCTCATCTCG-3′
plnfw2	5′-GAAGAAGCGACGCCGATCCAGTTGATGTGCCCCGA AGGTGCTATCTCGAAAG-3′
plnrev2	5′-CTTTTCAGGTTCTCCAGCGTGCGTG-3′
DHFRrevA	5′-GAAGTCTACGAGAAGAAAGACTCGAAGCTGTAACCC-3′
DHFRrevB	5′-GTCTACGAGAAGAAAGACTCGAAGCTGTAAGTCGACCCC-3′
DHFRfw	5′-CCCAAGCTTAAATAGGTTCGACCATTGAAC-3′

template.[17] The resulting *PLN* disruption cassette was transformed to *H. polymorpha* NCYC495 (*leu1.1 ura3*). Correct integration was confirmed by PCR and Southern blot analysis. The *pln.atg1* double mutant was constructed as follows. First an *atg1* deletion plasmid was constructed. Plasmid pBS-PDD7-URA[18] was digested with *Sal*I followed by self ligation, resulting in the generation of plasmid pKVK34. Subsequently, the 1.2 kb *Hin*dIII and *Nde*I fragment of pKVK34 (comprising nucleotides +1228 to +2414 of *ATG1*) was replaced by a 1.9 kb fragment of pB-LEU2Ca containing the *Candica albicans LEU2* gene (Genbank accession number AF000121) resulting in plasmid pKVK035. From this plasmid, a 3.2 kb deletion cassette was obtained by digesting the plasmid with *Dra*III-*Bgl*II, followed by transformation into *H. polymorpha pln* (*leu1.1).* Correct integration was analyzed by Southern blotting.

The PHIPX5-GFP-SKL plasmid[19] containing the GFP-SKL gene[20] under the control of *H. polymorpha* amine oxidase promoter (P_{AMO}) was linearized with *Bsi*WI and transformed to *H. polymorpha atg1* (*leu1.1*) (18) or *pln* (*leu1.1*) cells. Correct integration of a single copy of the gene was confirmed by Southern blot analysis.

For the subcellular localization of HpPln, a *GFP-PLN* hybrid gene under control of the *PLN* promoter (P_{PLN}) was constructed as follows: 1.26 kb of the *PLN* gene was amplified using the primers GFPplnfwA and GFPplnrevA introducing at the 5' end of the gene the 3' complementary overhang of GFP and a *Kni*t restriction site at the 3' end of the PCR product. The amplified 1.3 kb fragment (GFPplnrevB) was purified and used together with GFPplnfwB as primer for a second PCR reaction in which the GFPpln product was amplified and a *Bam*HI restriction site was introduced at the 5' end of the PCR product. For this purpose plasmid pHIPX5-GFP-SKL was used as a template. The hybrid gene was cloned as a *Bam*HI/*Kpn*I fragment into the corresponding sites of plasmid pHI1,[21] resulting in plasmid SSJ006, and transformed to *E.coli* DH5α. The promoter region of *PLN* was separately amplified using the primers GFPplnfwC and GFPplnrevC introducing a *Sph*I restriction site at the 5' end and a *Bam*HI site at the 3' end. The resulting PCR fragment was cloned as a *Sph*I/*Bam*HI fragment into the corresponding sites of plasmid SSJ006 yielding plasmid SSJ000. This plasmid was linearized with *Sal*I and transformed to *H. polymorpha* NCYC495 (*leu1.1 ura3*). Transformants containing a single copy integrated at the correct locus were selected using Southern blot analysis.

For colocalization studies, plasmid pHIPZ4-DsRed-SKL[22] was linearized with *Sph*I and transformed to WT:: P_{PLN}*GFP-PLN*. Zeocine resistant transformants were selected and analyzed for correct integration of the plasmid at the P_{AOX} locus by Southern blotting.

Construction of hybrid genes encoding *DHFR* variants. The hybrid genes encoding WT or mutant mouse dihydrofolate reductase (DHFR) with a C-terminal PTS1 (-SKL) were constructed. In the mutant DHFR protein Cys,[7] Ser,[42] and Asn[49] are replaced by Ser, Cys and Cys, respectively.[23,24] Genes encoding DHFR proteins containing a C-terminal -SKL were obtained by PCR using primers DHFRfw and DHFRrevA or DHFRrevB and the original DHFR plasmids. PCR products were isolated as a *Hin*dIII-*Sma*I and a *Hin*dIII-*Sal*I fragment respectively, and subcloned into pBluescript SK+(Stratagene, Amsterdam, NL). The plasmids containing WT (PJK001) or mutated DHFR (SSJ007) C-terminally tagged with -SKL were digested with *Hin*dIII-*Sma*I and *Hin*dIII-*Sal*I respectively and the isolated fragments were cloned into the corresponding sites of $PHIPX_4$ [25] resulting in plasmid PJK002 and SSJ001. These plasmids were linearized with *Sph*I and transformed into *H. polymorpha* NCYC 495 *leu 1.1* or *pln leu1.1.* Correct integration of a single copy of the plasmids at the P_{AOX} locus was determined by Southern blot analysis.

In vitro transcription/translation. The *AOX* gene was cloned as a *Bam*HI-*Sma*I fragment in the corresponding sites of the pBluescript II SK+ plasmid (Stratagene Inc., San Diego, CA), to allow transcription from the T7 promoter, resulting in plasmid SSJ007. In vitro ^{35}S-methionine labeled alcohol oxidase (AO) was synthesized from plasmid SSJ007, which was linearized with *Ssp*I. The TnT Coupled Wheat Germ Lysate System (Promega Corporation, Madison, WI) containing the T7 RNA polymerase was used together with ^{35}S-methionine (Amersham Pharmacia, Roosendaal, NL). In vitro synthesized ^{35}S-labeled protein was analyzed by SDS-PAGE and the cyclone storage phosphor image system (PerkinElmer, Boston, USA).

Cell fractionation. Cell fractionation was performed as described previously.[26] Post-nuclear supernatant fractions obtained from homogenized protoplasts were loaded onto sucrose gradients. The position of the peroxisomal peak fraction was determined by enzyme measurements of the peroxisomal marker enzyme AO.[27] Mitochondrial contamination in peroxisomal peak fractions (based on cytochrome c oxidase[28] measurements) were determined by established procedures and invariably below 5%. Peroxisomal matrix fractions were obtained from highly purified peroxisomes as described by Stewart et al.,[8] except that the peroxisomal fractions were resuspended in 500 µl 100 mM Tris, pH 8.0 to lyse the organelles followed by centrifugation for 15 min. at 200.000 x g (4°C) to remove peroxisomal membranes.

Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad GmbH, Munich, Germany) using bovine serum albumine as a standard.

Miscellaneous biochemical methods. Crude extracts were prepared as detailed before.[26] Proteolytic activity was assayed using ^{35}S-labeled in vitro translated AO oxidase as described by Stewart et al.[8] SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described.[29] Western blots were probed with polyclonal antibodies raised in rabbit against various *H. polymorpha* proteins and GFP or monoclonal mouse antibodies against DHFR (BD Biosciences,

Alphen aan de Rijn, The Netherlands). The chromogenic (NBT-BCIP) or chemiluminiscent (peroxidase, POD) Western blotting kit (Roche Diagnostics GmbH, Germany) were used. Quantification of GFP protein levels was performed by densitometric scanning of Western blots probed with anti-GFP antibodies.

Microscopy and flow cytometry. For electron microscopy and immunocytochemistry cells were fixed and prepared as described previously.[25] Cytochemistry to localize AO activities using the $CeCl_3$-based method, and catalase activities using 3'-3 diamino benzidine (DAB), was performed as detailed before.[30] Immunolabeling was performed on ultrathin sections of unicryl embedded cells using specific polyclonal antibodies against GFP or various *H. polymorpha* peroxisomal proteins and gold-conjugated goat-anti rabbit antibodies.[25] For the quantification of the number of peroxisomal profiles per cell section, random electron micrographs were taken of each sample; at least 150 cell sections were analyzed. Fluorescence microscopy was performed as described before.[31]

Reactive oxygen species (ROS) were visualized in living yeast cells using the ROS specific fluorescent dye dichlorodihydro-fluorescein diacetate (-H_2DCFDA; Molecular Probes, Invitrogen, Breda, The Netherlands). Cells were grown in batch cultures on methanol for 48 hours (late stationary growth phase), harvested by centrifugation and washed once in 50 mM potassium phosphate buffer pH 7.2. Then the cells were incubated in the same buffer containing 10 µM H_2DCFDA at 37°C for 30 minutes and washed again in the same phosphate buffer. The percentage of stained cells was determined by flow cytometry.

To determine cell viability, cells were extensively precultivated on glucose containing media and shifted to minimal media containing 0.5% methanol and grown for 16 days. Cells were harvested and incubated with 1 µM Sytox Green Nucleic Acid Stain (Molecular Probes, Invitrogen, Breda, The Netherlands) at 37°C for 30 minutes. After incubation the cells were washed once with demineralized water. The percentage of cells which stained positively with the fluorescent probe was determined by flow cytometry. For flow cytometric analysis of H_2DCFDA or Sytox Green stained cells a Beckman Coulter Epics XL-MCL was used at a low flow rate with excitation and emission settings of 488 and 525–550 nm, respectively. In each measurement at least 25000 events (cells) were analyzed. Samples of non-stained cells were used to correct for autofluorescence. The percentages of stained cells were expressed as averages from at least two independent experiments. Statistical analysis was performed by using the SigmaPlot software.

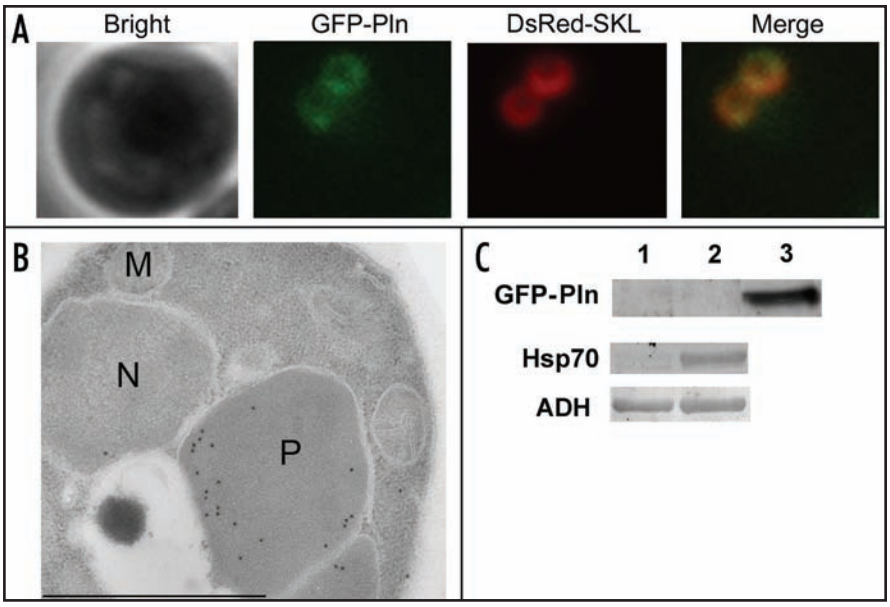

Figure 1. HpPln is localized in peroxisomes in *H. polymorpha*. (A) Bright field image, fluorescence and merged fluorescence images of *H. polymorpha* cells synthesizing GFP-Pln (green fluorescence) and DsRed-SKL (red fluorescence). Pln-GFP fluorescence colocalizes with DsRed-SKL in peroxisomes. (B) Immunocytochemical staining of the same cells using anti-GFP antibodies. The anti-GFP labeling is confined to peroxisomal profiles. M, mitochondrion; N, nucleus; P, peroxisome. The bar represents 0.5 µm. (C) Western blot analysis of crude extracts of *H. polymorpha* cells that produce GFP-Pln under control of the endogenous *PLN* promoter. The blots were probed with antibodies against the indicated proteins. In glucose-grown cells (lane 1) GFP-Pln fusion protein is not detectable, also not after heat shock treatment (lane 2), but the protein band is evident in methanol-grown cells (lane 3). The cytosolic heat shock protein Hsp70, used as a control for the heat shock treatment, was normally induced by heat shock. The constitutively expressed cytosolic enzyme alcohol dehydrogenase (ADH) was not induced by the heat shock. Equal amounts of protein were loaded per lane.

RESULTS

Identification of a peroxisomal Lon protease in *H. polymorpha*. A search in the genome database of the yeast *H. polymorpha*[32] revealed that this organism contains two putative Lon proteases, most likely one mitochondrial and one peroxisomal isoform. The putative peroxisomal Lon protease harbors a degenerate peroxisomal targeting signal type I (a C-terminal PTS1: -ARI-COOH). To determine the subcellular location of this putative peroxisomal Lon protease, a strain was constructed that expresses a GFP fusion protein under control of the homologous promoter. Fluorescence microscopy revealed that GFP fluorescence colocalizes with the fluorescent peroxisomal marker protein DsRed-SKL (Fig. 1A). The peroxisomal location of the protease was confirmed by immunocytochemistry using anti-GFP antibodies (Fig. 1B).

The putative peroxisomal Lon protease is a protein of 935 amino acids with a calculated mass of 104.9 kDa and shows 39% sequence identity with the putative peroxisomal Lon protease of *Mus musculus*. It contains an AAA-domain (aa 447–586) and a proteolytic domain (aa 665-857), which harbors the conserved active site serine residue (aa 789).[33]

We designated the encoding gene *H. polymorpha PLN* (peroxisomal Lon protease) and the corresponding protein HpPln. The *PLN* sequence was deposited at GenBank (Acc. No. DQ297145).

The same strain was used to analyze *PLN* expression at various growth conditions. Western blot analysis using anti-GFP antibodies revealed that synthesis of GFP-Pln is repressed during growth of cells on glucose, but induced on methanol, conditions that stimulate peroxisome proliferation (Fig. 1C). Expression of *PLN* was not significantly enhanced in glucose-grown cells upon heat shock (Fig. 1C), as reported for the mitochondrial Lon protease, PIM1, of *S. cerevisiae*.[34]

To analyze the function of HpPln, a strain was constructed in which the *PLN* gene was deleted (designated *pln*). *H. polymorpha pln* cells grew normal like WT on methanol at 37°C. Growth was also unaffected at elevated growth temperatures (45°C) or upon heat shock (data not shown).

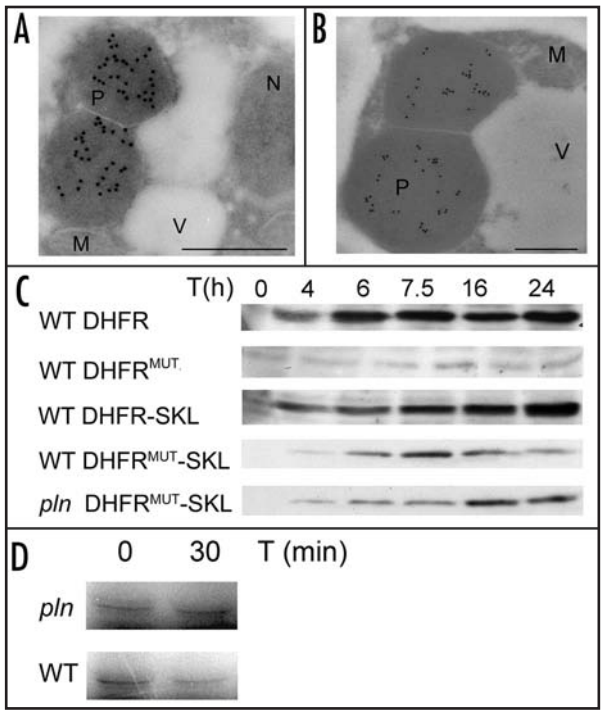

Figure 2. DHFR^{MUT}-SKL and AO monomers are stabilized in the absence of HpPln. (A and B) DHFR-SKL and DHFR^{MUT}-SKL are properly sorted to peroxisomes. Ultrathin sections of WT cells producing DHFR-SKL (A) or *pln* cells producing DHFR^{MUT}-SKL (B), grown for 16 hours on methanol, were labelled using anti-DHFR antibodies. Anti-DHFR labelling is confined to the peroxisomal profiles indicating that DHFR-SKL and DHFR^{MUT}-SKL were targeted to peroxisomes. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. The bar represents 0.5 μm. (C) shows Western blots probed with anti-DHFR antibodies of crude extracts of *H. polymorpha* WT or *pln* cells producing different DHFR variants. Cells were pregrown on glucose media and shifted (at T = 0 h) to methanol media to induce synthesis of the various DHFR proteins and HpPln. In WT cells DHFR and DHFR-SKL protein bands are first detectable four hours after the shift to methanol medium. The levels of these proteins gradually increased during prolonged cultivation. The cytosolic DHFR^{MUT} is also induced but remains invariably low, which can be explained by degradation of the protein by the Ub-proteasome pathway. The appearance of peroxisomal DHFR^{MUT}SKL is evident during the first 7.5 hours after the shift, both in WT and *pln* cells. Hence, peroxisome localized DHFR^{MUT}SKL protein is apparently more stable than cytosolic DHFR^{MUT}. In WT cells the levels of DHFR^{MUT}SKL, however, decrease again at later stages of cultivation (at T = 16 h and 24 h), which is not observed in the corresponding samples of the *pln* cells. These data indicate that peroxisomal DHFR^{MUT}SKL is stabilized in *pln* cells relative to WT controls. Western blots were prepared from crude cell extracts. Equal amounts of protein were loaded per lane. Experiments were performed at least twice. Representative blots are shown. (D) shows a Phospho-imager picture of an SDS-polyacrylamide gel showing the presence of ^{35}S-labeled in vitro synthesized AO protein upon incubation for 30 minutes at 37°C with purified peroxisomal matrix fractions isolated from *H. polymorpha pln* (upper panel) or WT cells (lower panel). The amount of AO protein decreased during incubation with WT peroxisome fractions, but not when *pln* derived peroxisomal fractions were used. Quantification of the bands revealed a decrease in AO signal to 51% of the original value at T = 0 h (set to 100%) in the AO sample incubated with WT peroxisome fractions, whereas no decrease in signal was detected when the protein was incubated with *pln* peroxisome fractions. Equal volumes of the incubation mixture were loaded per lane. The experiment was performed twice. A representative picture is shown.

HpPln degrades soluble unfolded and non-assembled peroxisomal proteins. To examine the role of HpPln in degradation of unfolded/misfolded proteins, we analyzed the fate of a mutant form of dihydrofolate reductase (DHFR) that contains three amino acid substitutions that destabilize the structure of the protein.[23] *H. polymorpha* strains were constructed that produce the WT (DHFR) or mutant variant of the protein (DHFR^{MUT}) containing a C-terminal PTS1 (-SKL). The genes were placed under control of the methanol inducible alcohol oxidase promoter (P_{AOX}). Immunocytochemistry (Fig. 2A and B) and cell fractionation experiments (data not shown), indicated that both proteins were properly sorted to peroxisomes.

The kinetics of the increase in levels of the two DHFR proteins was analyzed by Western blotting after a shift of cells from glucose to methanol (i.e., to conditions that induce P_{AOX}). The fate of cytosolic variants of both DHFR proteins was studied as well. The data presented in Figure 2C show that in WT cells the cytosolic DHFR protein was first detectable four hours after the shift to methanol medium. During the next hours the DHFR protein levels further increased and remained virtually constant upon prolonged cultivation. A similar pattern of induction was observed for peroxisome localized DHFR protein (DHFR-SKL) produced in WT *H. polymorpha*.

Upon induction of cytosolic DHFR^{MUT}, only minor amounts of DHFR^{MUT} protein were detectable at all time points after the shift. This most likely is due to degradation of newly synthesized DHFR^{MUT} protein by the cytosolic ubiquitin/proteasome system. Interestingly, higher levels were reached of the peroxisomal variant of the mutant DHFR protein (DHFR^{MUT}-SKL) both in WT and *pln* cells. Apparently, only a portion of the DHFR^{MUT}-SKL protein reaches the peroxisome, where it is protected from degradation by the proteasome (Fig. 2B). In WT cells, the highest levels of DHFR^{MUT}-SKL were detected at 7.5 h after the shift, followed by a decrease at later time points (at T = 16 and T = 24 h), which can be explained by the fact that *PLN* is also induced on methanol (see Fig. 1C). Indeed, a different pattern was observed in *pln* cells, in which the levels of DHFR^{MUT}-SKL further increased during prolonged growth.

Our data therefore indicate that peroxisome-localized DHFR^{MUT}-SKL protein was degraded in peroxisomes of WT cells , but relatively stable in peroxisomes of *pln* cells.

Previously, a peroxisomal protease has been identified and characterized in the related methylotrophic yeast *Candida boidinii*. This protease preferentially degraded in vitro synthesized alcohol oxidase (AO) monomers.[8] AO is imported into peroxisomes as FAD-containing monomers and assembles into enzymatically active octamers in the peroxisomal matrix.[35] In vitro synthesized AO does not bind FAD and remains monomeric.[36] We tested whether HpPln was capable of degrading in vitro synthesized AO protein by incubation of the protein in the presence of purified peroxisomal matrix fractions isolated from methanol-grown *H. polymorpha* WT or *pln* cells. The results, shown in Figure 2D, indicate that in vitro synthesized AO is degraded upon exposure to peroxisomal matrix proteins from WT cells, but not by matrix proteins of *pln* cells, suggesting that HpPln is capable of degrading non-assembled AO.

The absence of HpPln affects the viability of cells blocked in pexophagy. Next, we determined the percentage of dead cells in methanol-grown batch cultures after incubation in a shaker at 37°C for 16 days using flow cytometry of cells stained with the fluorescent stain Sytox. As shown in Figure 3, the percentage of dead cells in methanol-grown *pln* cultures was similar as that observed for WT controls, suggesting that HpPln is not important for the viability

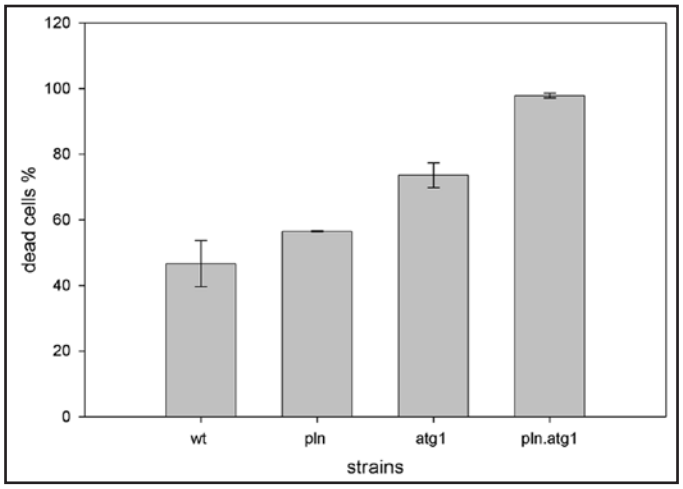

Figure 3. The absence of Atg1p and Pln results in decreased cell viability. The percentages of dead cells in WT, *atg1*, *pln* or *pln.atg1* cells obtained from flow cytometric analysis using Sytox stained cells are shown. The percentage of dead cells in *pln.atg1* cultures was significantly higher than that of WT, *pln* or *atg1* cells (student t-test p = 0.019, 0.0003 and 0.025, respectively). Cells were analyzed after 16 days of incubation in methanol containing medium at 37°C. The experiments were performed in duplicate. The error bars indicate the standard error of the two independent experiments.

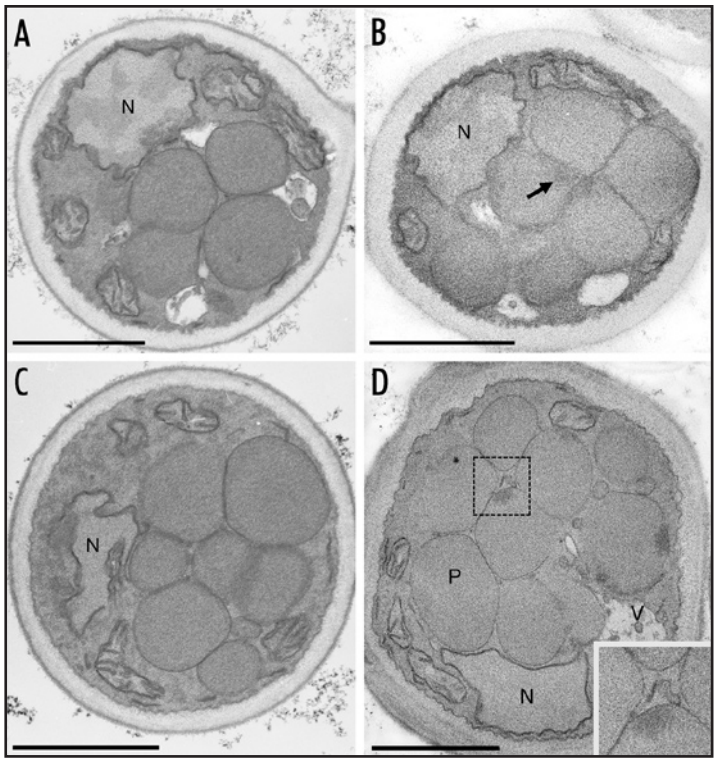

Figure 4. Morphology of WT, *pln*, *atg1* and *pln.atg1* cells. (A–D) show typical examples of peroxisomes in WT control (A), *pln* (B), *atg1* (C) and *pln.atg1* (D) cells. Organelles in *pln* cells (B and D) may contain electron dense aggregates (B, arrow). The magnification (D, inset) shows the indicated area in the cell that contains an aggregate. Cells were grown in a methanol-limited continuous culture (D = 0.1 h^{-1}) at 44°C. $KMnO_4$-fixation. N, nucleus; P, peroxisome; V, Vacuole. The bar represents 1 μm.

of *H. polymorpha* cells. We also analyzed the viability of *atg1* cells, which are unable to degrade peroxisomes by autophagy,[18] and cells of a *pln.atg1* double deletion strain. Our data revealed that the viability of *pln.atg1* cells was reduced relative to that of *atg1* cells (Fig. 3). Because the effect of the absence of HpPln on cell viability was very strong in an *atg1* background and less clear in a WT background, the negative effect of the absence of HpPln in *pln* cells may therefore be suppressed by degradation of whole peroxisomes by autophagy.

The absence of HpPln or Atg1p results in increased numbers of peroxisomes. Morphometric analysis of ultrathin sections of cells grown in methanol-limited chemostat cultures (dilution rate 0.1 h^{-1}) revealed that *atg1* cells harbor enhanced numbers of peroxisomes relative to WT controls (Fig. 4). The average number of peroxisomal profiles per cell section amounted 5.3 in *atg1* cells and 2.6 in WT controls (Fig. 5). These data indicate that during growth of WT cells on methanol peroxisomes proliferate but also may be constitutively degraded by autophagy. In *pln.atg1* double deletion cells the peroxisome abundance was comparable to that in *atg1* cells (average number of profiles 5.4; Figs. 4 and 5). Interestingly, in *pln* cells also a slight increase of peroxisome numbers was observed (average number of profiles per section 3.3; Figs. 4 and 5).

In *S. cerevisiae* mutants defective in the mitochondrial Lon protease (*PIM1*), characteristically results in the accumulation of protein aggregates,[34] which can be vizualized in electron micrographs as electron dense inclusions. Electron microscopy of methanol-limited chemostat grown cells revealed that in *H. polymorpha pln*—and infrequently in also in *atg1*—cells peroxisomes were observed to contain electron dense aggregates. These aggregates were most frequently observed in peroxisomes of *H. polymorpha pln.atg1* cells, but were invariably not detectable in peroxisomes of WT cells (Fig. 4).

Peroxisomes are constitutively degraded during vegetative growth of cells at peroxisome inducing conditions. Our findings that a block in autophagy (in *atg1* cells) results in enhanced numbers of peroxisomes suggests that peroxisomes are continuously formed and subsequently degraded again during cultivation of *H. polymorpha* WT cells on methanol. To analyse this in more detail, we determined the fate of WT peroxisomes using a methanol-limited chemostat at relatively low growth rate (D = 0.06 h^{-1}). A *H. polymorpha* strain was used, which contained the gene encoding GFP-SKL under control of the amine oxidase promoter (P_{AMO}). Synthesis of GFP-SKL was induced by growing the cells in media containing methylamine as sole source of nitrogen. After reaching the steady state, synthesis of GFP-SKL was subsequently fully repressed by adding excess ammonium sulphate to the culture and the feed. Fluorescence microscopy revealed that the peroxisomal GFP-SKL protein disappeared with time after repression of GFP-SKL synthesis by ammonium (Fig. 6). This reduction in GFP protein was confirmed by Western blot analysis of samples taken at various time points after repression of GFP-SKL synthesis. The decrease in GFP-SKL levels was faster than the theoretical wash-out curve of the culture (Fig. 6), indicating that GFP-SKL protein was actively degraded.

In *H. polymorpha atg1* cells the decrease in GFP-SKL protein levels was similar to the theoretical wash out curve of the chemostat culture, suggesting that the organelles are actively degraded in WT cells (Fig. 6). Similar experiments using *pln* cells indicated that HpPln did not significantly contribute to GFP-SKL degradation, because the decrease in GFP-SKL protein occurred with similar kinetics as observed in WT cells.

The Absence of Pln or Atg1p is Associated with Enhanced Cellular ROS Levels. Because peroxisomes are important sites of hydrogen peroxide production in methanol-grown *H. polymorpha* cells, we analyzed whether the observed strong decrease in cell

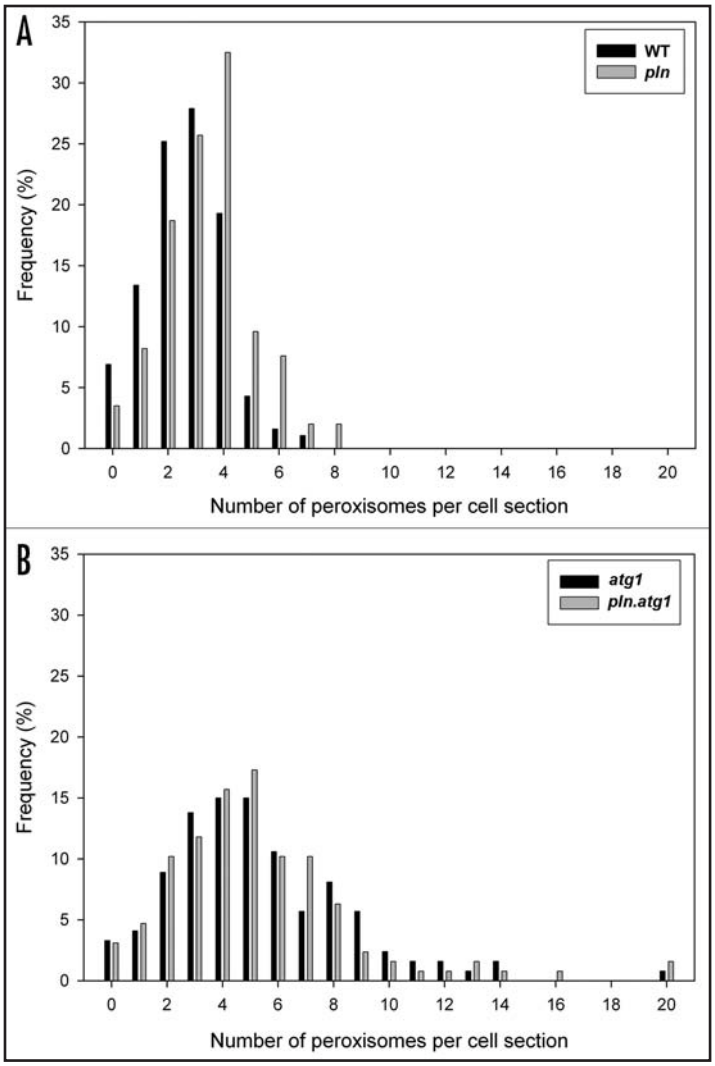

Figure 5. The number of peroxisomes is enhanced in *pln*, *atg1* and *pln. atg1* cells relative to *H. polymorpha* WT cells. Cells of WT, *pln*, *atg1* and *pln.atg1*were grown in a methanol-limited chemostat culture (D = 0.1 h^{-1}) at 45°C. Cells from steady state cultures were analyzed by electron microscopy. Electron micrographs were made from thin sections. For each strain the number of peroxisomal profiles per section was counted in electron micrographs of 150 randomly selected cell sections. The graphs show the frequencies (%) of sections with indicated numbers of peroxisomal profiles/cell section (A, WT and *pln*; B, *atg1* and *pln.atg1*). In most of the sections of WT cells 2–3 peroxisomal profiles were present and the maximum number was 7. In samples from *pln* cells most sections contained a slightly higher number of peroxisomal profiles (3–4) and the maximum number increased to 8. In *atg1* and *atg.pln* cells most sections contained 4–5 peroxisomal profiles, whereas the maxima increased to 20. Based on the data presented in the graphs the average numbers of peroxisomal profiles per section were calculated. These amounted 2.6 for sections of WT cells, 3.3 for *pln*, 5.4 for *atg1* and 5.3 for *pln.atg1*. The differences in average number are significant (t-test 99.9% confidence) between WT and *pln*, WT and *atg1* and WT and *pln. atg1*.

viability of *pln.atg1* cells is related to enhanced intracellular levels of reactive oxygen species (ROS). To this purpose we performed flow cytometry of cells stained with the ROS specific fluorescent dye H_2DCFDA. Cells were grown in batch cultures for 2 days in methanol containing media (and hence in the late stationary growth phase). As shown in Figure 7, the number of ROS containing cells had significantly increased in *pln.atg1* cells relative to WT control cells and the single deletion strains.

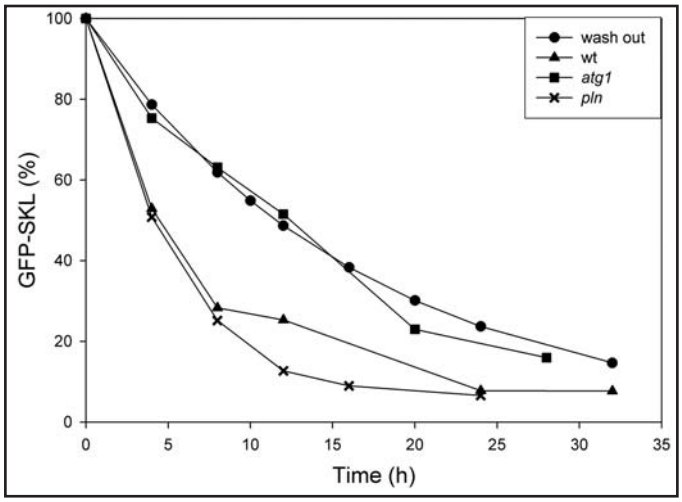

Figure 6. Decrease of GFP-SKL in *H. polymorpha* WT, *pln* and *atg1* cells grown in a methanol-limited chemostat culture. Cells of *H. polymorpha* WT (▲), *atg1* (■) and *pln* (x) expressing the gene encoding GFP-SKL under control of the amine oxidase promoter (P_{AMO}) were grown in continuous cultures at a dilution rate (D) of 0.06 h^{-1}. Samples were taken at indicated time points after full repression of GFP-SKL synthesis by excess ammonium. The decrease in GFP-SKL levels was monitored by Western blotting using anti-GFP antibodies. GFP protein levels were quantified by densitometric scanning of the blots. Equal amounts of proteins were loaded per lane. The sample at T = 0 h was set to 100%. The theoretical wash-out curve (●) was calculated from the dilution rate. The experiments were performed at least twice for each strain. Representative curves are shown.

The enhanced ROS levels may be related to reduced enzyme activities of peroxisomal catalase (CAT). However, Western blot analysis and enzyme measurements did not reveal a significant decrease in peroxisomal CAT protein or enzyme activities relative to WT controls (Table 4). We therefore analyzed the possibility that the enhanced organelle numbers was paralleled by an uneven distribution of CAT protein and/or enzyme activities over the peroxisomal population within one cell. CAT activity was stained using the standard procedure with DAB and hydrogen peroxide, generated by AO from methanol.[30] Cells were grown for two days in methanol media and hence in the late stationary growth phase. The results presented in (Fig. 8A) show that in WT controls all peroxisomes were positively stained for CAT activity upon incubation of cells in the presence of methanol (used as endogenous source for hydrogen peroxide production) and DAB. Identical experiments performed on *pln.atg1* cells (Fig. 8D) indicated that in the majority of the cells generally one or a few organelles showed reduced staining or no staining at all. The same phenomenon was also detectable in single *atg1* and *pln* cells, although to a lesser extend relative to *pln.atg1* cells (Fig. 8B and C). Control cytochemical experiments for the detection of AO activities, using the cerium based method, showed that in all strains all peroxisomes were positively stained for AO activity (shown for *atg1* and *pln*, (Fig. 8E and F; WT and *pln.atg1*, data not shown). However, the lack of CAT activity in a subset of the organelles was not due to the absence of CAT protein as shown by immunolabeling experiments using CAT antibodies. These experiments showed that CAT protein was present in all peroxisomes of methanol-grown *pln, atg1*, *pln atg1* and WT cells (shown for WT and *pln.atg1*, Fig. 8G and H; *atg1* and *pln*, data not shown). Therefore, our data suggest that the absence of Pln and Atg1 results in an imbalance in hydrogen peroxide production and removal.

Pln is a common protein in yeast and filamentous fungi. Finally, we analyzed whether peroxisomal Lon proteases are generally present in fungi. Gapped-Blast and Genomic Blast analyses were performed on fungal databases (December 2005) at the National Center for Biotechnology Information (NCBI) using primary sequences of fungal Lon proteases as queries.[37] This identified two Lon proteases in most fungi, one containing the peroxisomal targeting signal SRL-COOH or a derivative thereof, while the other has a putative N-terminal mitochondrial targeting signal. Remarkably, peroxisomal Lon proteases were not detected in the yeast species *S. cerevisiae* and *Candida glabrata*, although the mitochondrial isoforms could readily be identified in these organisms. This is even more surprising since the genome of the closely related species *Kluyveromyces lactis* and *Ashbya gossypii* encode both peroxisomal and mitochondrial Lon proteases, suggesting that the gene encoding the peroxisomal isoform may have been lost in *S. cerevisiae* and *C. glabrata* during evolution.

Phylogenetic analysis of plant, mammalian and fungal Lon proteases of various origins, using the Lon protease of *Escherichia coli* (protease La) as out-group, confirmed the existence of two phylogenetic groups in eukaryotes, which probably have a common ancestor (Fig. 9).

Table 4 **Catalase protein levels and enzyme activities in WT and various mutant strains**

	WT	*pln*	*atg1*	*pln.atg1*
CAT protein (arbitrary units)	9.1 ± 0.7	10.5 ± 0.7	11.0 ± 1.0	9.6 ± 0.2
CAT activity ($\Delta E_{240}.min^{-1}.mg^{-1}$)	61.7 ± 10.4	83.4 ± 7.3	77.4 ± 2.5	81.4 ± 3.7

Cells were grown for two days in methanol containing batch cultures (i.e., to the late stationary growth phase). The level of CAT protein was determined by densitometric scanning of Western blots probed with anti-CAT antibodies. Equal amounts of protein (total cell lysates) were loaded per lane. CAT enzyme activities were measured in cell free extracts prepared from the same cells. Averages are given from two independent experiments. The standard error was calculated using Sigma Plot. The data indicate that relative to the WT control neither the CAT protein levels nor the CAT activity levels significantly decreased.

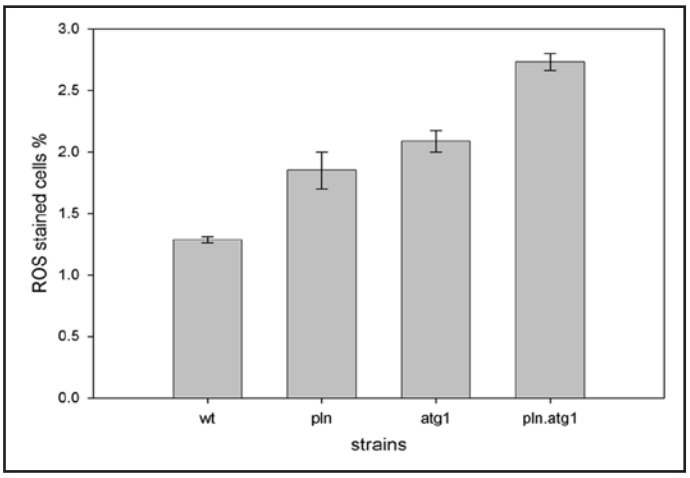

Figure 7. The absence of Atg1p or Pln causes enhanced intracellular reactive oxygen species (ROS). The percentage of cells showing intracellular levels of reactive oxygen species (ROS) that could be visualized by the ROS specific dye H_2DCFDA determined by flow cytometry. The number of positively stained cells was enhanced in *pln, atg1* and *pln.atg1* cells relative to WT controls (student t-test, p = 0.065, 0.012 and 0.003, respectively). Also a significant difference was observed between *atg1* and *pln.atg1* cells (p = 0.028). Cells were analyzed after two days of incubation in methanol-containing medium in a shaker at 37°C. The experiments were performed in duplicate. The error bars indicate the standard error of the two independent experiments.

DISCUSSION

Here we report on the identification and function of a peroxisomal Lon protease, HpPln, in degradation of peroxisomal matrix proteins in the yeast *H. polymorpha*.

Lon is a highly conserved ATP-stimulated protease, which belongs to the family of AAA-ATPases. The ATPase domain in Lon proteases is required for ATP-dependent unfolding of the target protein, prior to degradation by the protease domain.[38] Lon proteases from bacteria and mitochondria in eukaryotes have been extensively studied. Only recently a peroxisomal Lon protease was identified in mammals.[7] We now report for the first time on a peroxisomal Lon type protease in yeast. The analysis of available databases revealed that peroxisomal Lon proteases are very common in fungi. However, *S. cerevisiae* seems to be an exception to this rule. Why this organism and also the closely related yeast *Candida glabrata* lack a peroxisomal Lon protease is unknown.

The first identified member of the family of Lon proteases is *E. coli* protease La.[39] This protease is involved in the degradation of short-lived and abnormal proteins, specifically those produced under stress conditions. The mitochondrial Lon protease in *S. cerevisiae* (PIM1) is important for turn-over of misfolded and non-assembled mitochondrial matrix proteins. Cells of an *S. cerevisiae PIM1* deletion strain are respiratory deficient and unable to utilize non-fermentable carbon sources. Due to the impaired capacity to degrade misfolded matrix proteins, protein aggregates accumulate in mitochondria of *pim1* cells.[34]

Our data suggest that HpPln is a major protease involved in degradation of non-functional peroxisomal matrix proteins, because both $DHFR^{mut}$-SKL and monomeric AO are stabilized in the absence of HpPln. Moreover, in *H. polymorpha pln* cells aggregates accumulate in the organelle matrix, suggesting that the function of HpPln to remove unfolded protein is not taken over by other peroxisomal proteases.

In peroxisomes of *C. boidinii* a peroxisomal protease has been identified that specifically degrades in vitro synthesized monomeric AO.[8] Biochemical characterization revealed that this protein is an oligomeric serine protease with a native molecular weight of 80–90 kDa. The corresponding gene was not isolated, but the properties of the enzyme make it very unlikely that it represented the *C. boidinii* homologue of HpPln.

Another mode of proteolytic degradation of peroxisomal proteins is the turnover of whole organelles by autophagy. In *H. polymorpha* selective peroxisome degradation is known to be induced upon repression of methanol-metabolism, e.g., by shifting methanol-grown cells to glucose- or ethanol-containing media.[11,40] The physiological reason behind this phenomenon is that at these conditions most peroxisomal enzymes (e.g., AO) become redundant for growth. A non-selective mode of peroxisome degradation by autophagy is induced in yeast cells during nitrogen starvation, which triggers non-selective turnover of cellular content.[41]

We now demonstrate that in WT *H. polymorpha* cells peroxisomes —or at least a subset of the organelles—have a relatively short half

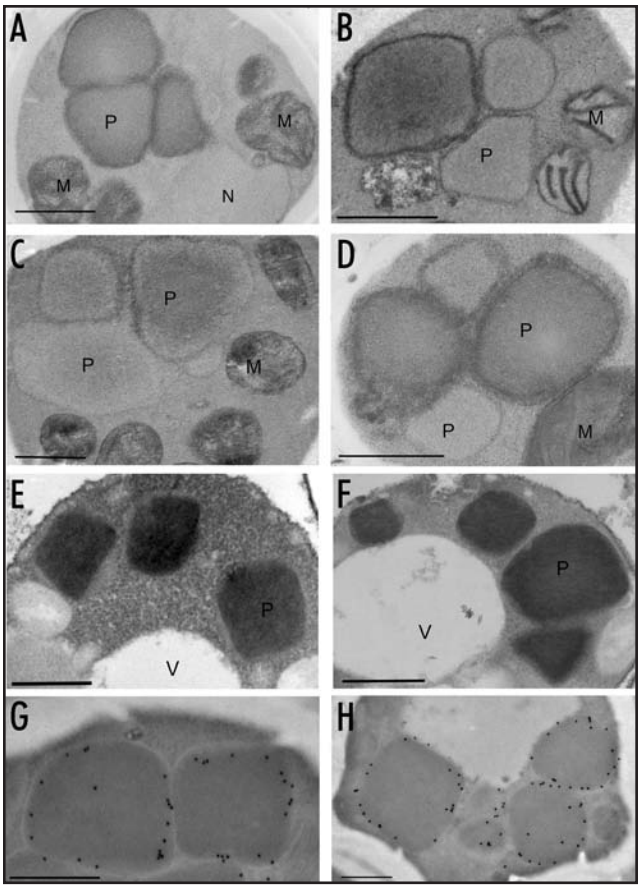

Figure 8. Cytochemical staining for catalase (DAB) and alcohol oxidase activity. Catalase (CAT) activity was stained using 3-3′ diamino benzidine (DAB) and hydrogen peroxide, generated from methanol by alcohol oxidase (AO) activities. Cells were incubated in the presence of 2 mg/ml DAB and 20 mM methanol for 2 hours and post fixed with $KMnO_4$. WT cells contain positively stained peroxisomes (Fig. 8A), while in a representative cell of the *pln.atg1* culture (Fig. 8D) two out of four organelles stain together with two organelles that are hardly (upper organelle) or not at all stained (lower organelle). The reduction in number of CAT stained organelles relative to WT controls was also evident in *pln* (Fig. 8B) and *atg1*(Fig. 8C) cells, but in these cells the frequency of CAT negative organelles was lower as compared to the *pln.atg1* cells. Note that the staining accumulates at the non-crystalline edge of the organelles, where CAT is predominantly located in *H. polymorpha* peroxisomes (see also Figs. 8G, H). Mitochondria are also stained because of cytochrome c peroxidase activity. Control cytochemical experiments for AO activity showed that invariably all organelles showed AO activity in WT, *atg1*, *pln* and *pln.atg1* cells (shown for *atg1*and *pln*; Figs. 8E, F). Immunolabeling with anti-CAT antibodies showed that CAT protein was present in all peroxisomes in cell of all strains under study (shown for WT, Fig. 8G, and *pln.atg1*, Fig. 8H) (*atg1* and *pln*; data not shown). The bars represent 0.5 µm. M - mitochondrion, N - nucleus, P - peroxisome, V - vacuole.

life and are constitutively degraded by autophagy also at normal, vegetative growth conditions (i.e., at nitrogen excess conditions and no change in carbon source). As a result peroxisome abundance significantly increases upon preventing autophagy by deletion of the *ATG1* gene. Constitutive autophagy of peroxisomes may therefore represent another important means to prevent accumulation of non-functional peroxisomal proteins/peroxisomes in yeast cells as part of the cellular housekeeping machinery.

Our data indicate that the constitutive peroxisome degradation may suppress the negative effects of the absence of HpPln (in *pln* cells). In an *atg1* background, the absence of HpPln resulted in a strong

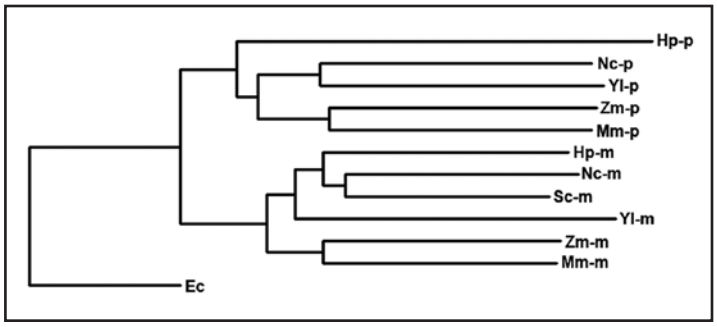

Figure 9. Phylogenetic tree of putative Lon proteases. *Escherichia coli* (Ec, GenBank Accession Number AAC36871), the mitochondrial isoforms of *Mus musculus* (Mm-m, GenBank AAN85210), *Zea maize* (Zm-m, Swissprot P93648), *H. polymorpha* (Hp-m, GenBank DQ341384), *Saccharomyces cerevisiae* (Sc-m, embl CAA84841), *Yarrowia lipolytica* (Yl-m, embl CAG78709) and *Neurospora crassa* (Nc-m, GenBank EAA32590) and the putative peroxisomal ones of *M. musculus* (Mm-p, GenBank AAH49090), *Z. maize* (Zm-p, GenBank AAC50011), *Y. lipolytica* (Yl-p, embl CAG78606), *N. crassa* (Nc-p, GenBank EAA33280) and *H. polymorpha* (Hp-p, GenBank DQ297145). *E. coli* was used as an out-group species. The data show that eukaryotic Lon proteases form two phylogenetic groups, one containing the mitochondrial isoforms, the other the peroxisomal ones.

decrease in cell vitality, indicating that timely removal of unfolded matrix proteins thereby preventing the formation of peroxisomal protein aggregates is very important for the viability of cells. Proper degradation of unfolded proteins in the cytosol[42] or mitochondria[43] of eukaryote cells is known to be very important for cell viability. Unfolded proteins are often highly toxic because of their tendency to form sticky, intracelullar protein aggregates. The accumulation of intracellular protein aggregates is also one of the key pathological features of various human neurogenerative diseases. Our current findings indicate that also the removal of unfolded proteins in the peroxisomal matrix and prevention of the formation of intra-organellar aggregates is important for cell viability. Possibly, peroxisomal CAT is predominantly affected by the toxic effect of peroxisomal aggregates, as suggested by our finding that in the absence of Atg1p and/or Pln some of the organelles show reduced CAT activities.

In *pln* cells the average peroxisome numbers slightly increased relative to WT controls. The most likely explanation for this is that peroxisome proliferation is stimulated in these cells. Interestingly, Legakis et al[44] also observed an increase in peroxisome abundance in ageing human cells, suggesting that the accumulation of damage inside peroxisomes may stimulate organelle proliferation. This may imply that the cells can recognize damage/malfunctioning of specific compartments. Studies to evaluate this possibility are currently underway.

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